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Pharmacodynamic studies of nasal tetracosactide with salivary glucocorticoids for a non-invasive Short Synacthen Test

Dr Charlotte J. Elder[1,2], PhD, Ruben Vilela[1], BMedSci, Dr Trevor N. Johnson[3], PhD, Rosie N Taylor[4], MSc, Dr E. Helen Kemp[1], PhD, Prof Brian G. Keevil[5], PhD, Dr Alexandra S. Cross[1], MBChB, Prof Richard J. Ross[1], MD, and Dr Neil P. Wright[2], MD.

[1]Department of Oncology and Metabolism, The University of Sheffield, Sheffield S10 2RX, United Kingdom; [2]Department of Endocrinology, Sheffield Children's NHS Foundation Trust, Sheffield, Western Bank, Sheffield S10 2TH, United Kingdom; [3]Certara UK Limited, Concourse Way, Sheffield, S1 2BJ, United Kingdom; [4]Statistical Services Unit, The University of Sheffield, Sheffield S3 7RH, United Kingdom; [5]Department of Clinical Biochemistry, Manchester Academic Health Sciences Centre, Manchester University NHS Trust, Southmoor Rd, Manchester M23 9LT, United Kingdom;

Corresponding author (and for reprint requests):

Dr Charlotte Elder,

Academic Unit of Child Health,

Sheffield Children's Hospital,

Western Bank,

Sheffield S10 2TH,

United Kingdom.

Tel: +44 114 305 3331

Email: c.j.elder@sheffield.ac.uk

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Nasal Tetracosactide: non-invasive Synacthen Test

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Disclosure summary: CJE and NPW have a patent application for Nasacthin003. RJR is a Director of Diurnal Group Plc. and holds shares. TNJ holds shares in Diurnal Group Plc. RV, RNT, EHK, BK and ASC report no conflicts of interest in this work.

Abbreviations: AI, adrenal insufficiency; AUC, area under the curve; CV, coefficient of variation; EMA, European Medicines Agency; LCMS, liquid chromatography mass

47 spectrometry; PD, pharmacodynamic; PK, pharmacokinetic, SmPC, Summary of Product
48 Characteristics; SST, Short Synacthen test.
49
50

Abstract

Context: The Short Synacthen Test (SST) is the gold standard for diagnosing adrenal insufficiency. It requires invasive administration of Synacthen, venous sampling and is resource-intensive.

Objective: To develop a nasally administered SST, with salivary glucocorticoids measurement, to assess the adrenal response.

Design: We conducted five studies: four open-label, sequence-randomised, crossover, pharmacodynamic studies testing six doses/formulations and a repeatability study. Additionally, pharmacokinetic analysis was undertaken using our chosen formulation, 500 µg tetracosactide with mucoadhesive chitosan, Nasacthin003, in our paediatric study.

Setting: Adult and children's Clinical Research Facilities.

Participants: 36 healthy adult males and 24 healthy children.

Intervention: We administered all six nasal formulations using a CE marked atomisation device. The intravenous comparators were 250 µg or 1 µg SST.

Main Outcome Measures: We analysed paired blood and saliva samples for plasma cortisol and salivary cortisol and cortisone.

Results: The addition of chitosan to tetracosactide and dose escalation increased peak cortisol response (p=0·01 and 0·001 respectively). The bioavailability of Nasacthin003 was 14·3%. There was no significant difference in plasma cortisol at 60 minutes between 500 µg Nasacthin003 and 250 µg intravenous Synacthen (p=0.17). The repeatability coefficient at 60 minutes was 105 nmol/l for intravenous Synacthen and for salivary cortisol and cortisone was 10·3 and 21·1 nmol/L respectively. The glucocorticoid response in children was indistinguishable from that of adults.

Conclusions: Nasal administration of Nasacthin003 generates equivalent plasma cortisol values to the 250 µg intravenous SST and, with measurement at 60 minutes of salivary cortisol or cortisone, provides a non-invasive test for adrenal insufficiency.

Introduction

The Short Synacthen Test (SST), or ACTH (cosyntropin) stimulation test, is the most commonly used diagnostic test for adrenal insufficiency (AI) and the recommended, gold standard test for primary AI.[1,2,3] The test involves either intravenous or intramuscular injection of synthetic ACTH(1-24) (tetracosactide) and blood sampling at 30-60 minutes to quantify the plasma cortisol response. Use of the test is increasing.[3,4] For most healthcare providers the test is time-, labour- and resource-intensive. Cannulation (or administration of intramuscular Synacthen) and venous sampling may be distressing and painful, especially for children. These barriers may lead to delayed or missed diagnoses, with a risk of death through an adrenal crisis.[5] There is a need for a less invasive, less resource-intensive, therefore more cost-effective, test for AI.

The label for Synacthen recommends a 250 µg intravenous dose; however, in clinical practice, both a high- and low-dose test are used as 250 µg provides a supraphysiological dose of Synacthen.[3] Meta-analyses demonstrate similar outcomes for both the high- and low-dose test.[6,7] There is a threshold tetracosactide level that elicits a maximal cortisol response at 30 minutes and this is achieved with 1 µg intravenous Synacthen, with higher doses generating more prolonged stimulation.[8-10] Salivary cortisol is a validated and well established alternative to invasive glucocorticoid sampling.[11] It has been investigated as an alternative to plasma cortisol following the administration of Synacthen in both healthy volunteers and patient populations.[12-14] Salivary cortisone is emerging as the preferable salivary biomarker as it is more abundant in saliva than cortisol, more sensitive at low plasma cortisol levels, and better reflects plasma total and free cortisol than salivary cortisol.[12-16]

We have investigated alternative routes for Synacthen administration. The intranasal route has advantages: minimal training to administer drugs in a rapid and tolerable way; good absorption due to the richly vascular nasal mucosa; avoidance of first pass metabolism; and a rapid onset of action.[17] The side effects of intranasal drugs are few and are generally attributable to the drug itself rather than the method of delivery. Previous studies have examined the potential of nasally administered ACTH analogues but as a replacement for depot ACTH, historically used as an alternative to corticosteroid treatment in inflammatory conditions. Despite a demonstrable adrenal response, the short duration of Synacthen activity limited their therapeutic advancement.[18,19] We have developed an intranasal formulation of Synacthen and tested it in both adults and children, with the aim of generating a non-invasive test.

Materials and Methods

Studies design and participants

We conducted five studies (S1-S5) at the Clinical Research Facilities of Sheffield Children's NHS Foundation Trust and Sheffield Teaching Hospitals NHS Trust, UK between 2010 and 2017. Studies S1-3 and S5 were open-label, multi-arm, sequence-randomised, crossover, pharmacodynamic (PD) studies. S4 was a repeatability study. Studies S1-S4 were conducted in healthy adult males and study S5 in healthy children. As a "first in man" study, ethical restrictions precluded administration of the novel drug product to women of childbearing age. The numbers assessed for eligibility, recruited, completing study visits and included in data analyses for each study are displayed in the CONSORT table (table 1). We recruited 12 different adults to each of the pharmacodynamic studies, with six volunteers from the dose-response study (S3) re-

enrolled for the repeatability study (S4), and 24 children participating in the paediatric study (S5). We excluded volunteers if they smoked, had been diagnosed with an endocrinopathy, intra-cranial or adrenal pathology, asthma, allergic rhinitis, anaemia, peptic ulcer disease, gastrointestinal bleed or dyspepsia, experienced a severe allergic reaction or any hypersensitivity to synacthen, were on any regular or prescribed medication, received any formulation of corticosteroid in the previous three months or had ever had a course of oral corticosteroids lasting more than one month. The first study was approved by Leeds (West) Research Ethics Committee, UK and all subsequent studies by London-Hampstead Research Ethics Committee, UK. Written informed consent was given by all participants or their parents/carers. The studies were registered with EudraCT (2009-013355-29 and 2012-003241-15).

Nasal tetracosactide formulations

Our initial study (S1) used 25 µg and 100 µg of the licenced intravenous Synacthen formulation (250 µg/ml, Alliance Pharmaceuticals Wiltshire, UK) which we administered intranasally. Doses were based on the results of a murine study but were poorly absorbed.[20] For subsequent studies the tetracosactide was specifically manufactured (Archimedes Pharma, Nottingham, UK) presenting us with the opportunity to formulate with concentrations of tetracosactide (Bachem AG, Bubendorf, Switzerland) in volumes suitable for nasal administration (0·1-0·2 ml per nostril) and with chitosan (FMC BioPolymer AS, Sandvika, Norway), a drug enhancer to optimise nasal absorption. Chitosan, a polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine, is derived by partial deacetylation of chitin from crustacea. It is a cationic biopolymer, acting as a bioadhesive film-forming agent to increase drug residence time in the nose, slow mucociliary clearance, and may

facilitate paracellular transport of large polar molecules. It is not systemically absorbed and has an excellent safety profile, with a multitude of applications including clarification agent, fungicide, nutritional supplement, and cosmetics constituent.[17]

The nasal formulations we chose for the optimisation study (S2) examined the effects of chitosan addition, dose escalation and the additive effect of both. The nasal formulations were: Nasacthin001 containing 100 µg tetracosactide with chitosan (0·2 ml of 0·5 mg/ml), Nasacthin002 containing 500 µg tetracosactide (0·2 ml of 2·5 mg/ml), and Nasacthin003 containing 500 µg tetracosactide with chitosan (0·2 ml of 2·5 mg/ml). Nasacthin003 was subsequently given in double dose (0·4 ml of 2·5 mg/ml) to yield 1000 µg tetracosactide with chitosan.

Procedures

All studies were carried out employing a similar methodology. Participants were given 1 mg (0·5 mg for under 8-year olds) dexamethasone the night before and the morning of each visit to establish a uniform glucocorticoid baseline and to allow for plasma tetracosactide quantification. We verified adherence by low or undetectable plasma cortisol (<50 nmol/L) on a baseline sample. Additionally in study 1 (S1) we tested -1 minute (baseline) samples for plasma ACTH on an immunochemiluminmetric assay (Immulite 2000, Siemans Healthineers, Munich, Germany), verifying adherence as an ACTH level of <5 IU/L.

All visits commenced before 09.30. Volunteers rested for 30 minutes following intravenous cannulation and remained supine throughout. Participants attended for nasal tetracosactide visits or the intravenous comparator visit (high-dose Synacthen (250 µg, 12 adults; 145 µg /m^2, 12 children) or low-dose (1 µg, 23 adults, 12 children))

in a randomised order, with no fewer than seven days between visits. Our dilution method for low-dose Synacthen has been described previously.[14] We administered nasal preparations via a readily available, CE marked Mucosal Atomisation Device (Teleflex®, Wayne, PA, USA), 0·1 ml to each nostril (0·2 ml for 1000 µg dose). Paired blood, taken from the indwelling cannula, and saliva samples, a minimum of 1 ml collected by passive drool into a Salicap tube (IBL, Hamburg, Germany), were taken at the following times (administration of tetracosactide at 0 minutes): -15, -1, 2, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, and 120 minutes. We requested participants rinse their mouths thoroughly with water ten minutes before the first salivary sample and not to eat or drink anything, other than water, until after the final sample collection. Participants initiated the salivary drool at the same time the syringe was connected to the cannula to withdraw the discard, prior to blood sampling.

The six participants we re-recruited from the dose response study (S3) to participate in the repeatability study (S4) underwent two further SST using 500 µg Nasacthin003 with sampling times at -1, 5, 10, 30, 40, 60 and 90 minutes. In the paediatric study (S5) we recruited 24 children, all of whom received the 500 µg Nasacthin003 formulation for their intranasal visit, but were randomised to receive either high-dose (145 µg/m^2) or low-dose (1 µg) Synacthen as their intravenous comparator. After completion of their visits, participants were invited to complete a post-study questionnaire to gauge their personal experience of the intravenous and nasal tests.

We froze and stored samples, batch analysing at the end of each of the five studies. There were no changes to the assay platforms between studies. We analysed plasma cortisol samples using the Abbott Architect i1000 chemiluminescent microparticle immunoassay (Abbott Diagnostics Ltd, Berkshire, UK). Our salivary cortisol and cortisone analyses were performed by a modified liquid chromatography-tandem mass

spectrometry (LC-MS/MS) assay using a Waters Xevo TQ-MS mass spectrometer and a Waters Acquity LC system with an electrospray source operated in positive-ionization mode. We have reported assay characteristics previously.[14] In study S5 we measured plasma tetracosactide levels by an ACTH(1-24) EIA Kit (Peninsula Laboratories International, Inc., San Carlos, CA, USA). Assay sensitivity, 72 pg/ml; intra-assay precision, 2·73% (at 165 pg/ml) and 1·76% (at 400 pg/ml); and inter-assay precision 9·32% (at 160 pg/ml) and 6·50% (at 412 pg/ml). The EIA ACTH(1-24) data sheet reports 100% cross-reaction with ACTH(1-39) in the assay.

Randomisation

We randomised formulation to participant visits in studies S2, S3 and S5 using an online randomisation programme. The randomisation used a block permutation method which created a balanced randomisation. The randomisation plan was produced for 20 participants to allow for further volunteers to be randomised should any of the original recruits drop out.

Outcomes

Our primary outcome was to develop a non-invasive alternative to the intravenous SST. Our secondary outcomes were: comparison of plasma cortisol at different time points between the nasal and intravenous formulations; comparison of the response both within and between participants to assess repeatability; comparison of the response between children and adults; and (in the paediatric study only) comparison of the pharmacokinetic parameters (time to maximum plasma concentration (Tmax),

maximum plasma concentration (Cmax), area under the concentration time curve (AUC) and bioavailability) achieved.

Statistical analysis

We selected the sample size for each study in accordance with EMA bioavailability study guidance.[21] Only participants who were adequately dexamethasone-suppressed and completed at least one nasal and the intravenous comparator visit were included in the final analyses (table 1). Our safety analysis includes all participants who received nasal formulations. We examined the plasma cortisol response to each formulation over time for each participant. In order to mirror the most popular sampling times following intravenous Synacthen we truncated cortisol response over time graphs at 60 minutes.[3,4] We used descriptive statistics, mean and standard deviation (SD) to describe the plasma cortisol, salivary cortisol and salivary cortisone response to each formulation of tetracosactide. Where participants had both formulations we used paired t-tests and where the formulations had been given to different participant groups we used independent samples t-tests to compare the difference of the peak plasma cortisol, 30-minute and 60-minute cortisol between the intravenous comparator and the nasal formulation. A p value of less than 0·05 indicated a statistically significant difference. We used coefficient of variation (CV) to quantify the variability between formulations, between-participants and within-participants and standard deviations to further examine between-participant variability. Additionally, we assessed the within-participant repeatability using data from study S4 to calculate a 30- and 60-minute repeatability coefficient. The repeatability coefficient is calculated from the within participant standard deviation (s_w) as $1.96\sqrt{2}\ s_w$ and describes the range within which

two observations on the same individual would be expected to fall 95% of the time. We used SAS v9·3 for statistical analyses.

Pharmacokinetic (PK) analysis of Tetracosactide data in paediatric study (S5):

We calculated the PK parameters: Tmax, Cmax, AUC from time zero until the last quantifiable time point (AUC_{0-t}) and AUC from time zero until infinity ($AUC_{0-\infty}$) for each individual using non compartmental analysis in Phoenix WinNonLin 6·4. For determination of AUC values we used the linear up and log down trapezoidal method. For $AUC_{0-\infty}$ the terminal slope (Lambda Z) of the concentration-time profile was determined using the 'Best fit' method in WinNonLin. We obtained descriptive statistics for the PK parameters for the intravenous and nasal tetracosactide formulations. The absolute bioavailability of the Nasacthin003 was calculated based on $AUC_{0-\infty}$ compared to the intravenous Synacthen 250 µg dose.

Results

Participants: We enrolled the first participant to study S1 on July 7, 2010 and the last to study S5 on August 17, 2017. The numbers screened for eligibility, participating in each study and included in the final analyses are displayed in table 1. The adult studies (S1-S4) recruited 36 healthy males, aged 19-46 years (median 22, IQR 21·5-23·0), with BMIs of 19·1-29·4 kg/m^2 (median 23·2, IQR 21·7-24·4). There was no indication that BMI influenced the efficacy of the nasal formulations. In the paediatric study (S5) we recruited 36 children but 12 did not complete their initial visit due to unsuccessful cannulation or difficulties obtaining samples and did not continue in the study. Thus,

24 healthy children (12F) participated, aged 5-14 years (median 10·5, IQR 9·0-12·5), with BMIs between the 4th-93rd centiles (median 51·5, IQR 31·5-74·0).

Formulation development and optimisation (table 1 and 2): These were based on the pharmacodynamic cortisol response to Synacthen. Our initial study (S1) used a commercially available intravenous formulation of Synacthen administered intranasally at 25 and 100 µg and the peak cortisol response was significantly lower when compared to the 1 µg intravenous comparator (mean difference and 95% confidence interval: -320 nmol/L (-370, -271) and -222 nmol/L (-297, -146), p<0·0001 and p=0·0001 respectively). We then tested three novel intranasal formulations (study S2) to examine the effect of dose escalation and the addition of the mucoadhesive chitosan: Nasacthin001 (100 µg tetracosactide with chitosan), Nasacthin002 (500 µg tetracosactide) and Nasacthin003 (500 µg tetracosactide with chitosan). The peak cortisol responses showed an increase with the addition of chitosan with mean difference (95% confidence interval): 177 nmol/L (54, 299) p=0·007 for 100 µg formulation and 222 nmol/L (73, 370) p=0·01 for 500 µg formulation. The increase in dose from 100 to 500 µg also significantly increased the cortisol response: 242 nmol/L (112, 372) p=0·0011 for formulations without chitosan and 339 nmol/L (137, 541) p=0·0063 for formulations with chitosan. We therefore selected to progress with formulation Nasacthin003 (500 µg tetracosactide and chitosan) for studies S3, S4 and S5 as it gave the maximal cortisol response.

Nasacthin003 compared to intravenous Synacthen (table 3, figure 1): We compared 250 µg intravenous Synacthen with Nasacthin003 at 500 µg and 1000 µg in study S3.

As can be seen in figure 1a the mean plasma cortisol concentrations of all three formulations are similar up to 60 minutes. There was no significant difference in the 60-minute plasma cortisol between either the 500 µg or 1000 µg Nasacthin003 nasal formulation compared with 250 µg intravenous Synacthen, with mean difference (95% confidence interval): -28 nmol/L (-70, 14) p=0·17 and -16 nmol/L (-48, 16) p=0·30, respectively. For salivary cortisol and cortisone the shape of the curve was similar following administration with both 250 µg intravenous Synacthen and Nasacthin003 (figure 1b, 1c). However, the salivary cortisol and cortisone levels at 60 minutes were lower following intranasal administration. The mean difference (95% confidence interval) for salivary cortisol and cortisone at 1000 µg was -1·5 nmol/L (-3·8, 0·7) p=0·16 and -5·2 nmol/L (-7·5, -2·8) p=0.0005 respectively and at 500 µg was -4·5 nmol/L (-8·3, -0·6) p=0·028 and -8·4 nmol/L (-15·2, -1·6) p=0·02, respectively.

Reproducibility: The between-participant variability of cortisol response at 60 minutes, represented by the standard deviation, calculated using all participants who received 500 µg Nasacthin003 (n=46, excluding repeated observations), was 95 nmol/L for plasma cortisol, 7 nmol/L for salivary cortisol and 14 nmol/L for salivary cortisone. The CVs were 18%, 32%, and 24% respectively and were significantly different (all pairwise comparisons p<0.0001). For participants receiving 250 µg intravenous Synacthen (n=23) the standard deviation was 60 nmol/L for plasma cortisol, 7 nmol/L for salivary cortisol and 12 nmol/L for salivary cortisone. The CVs were 11%, 30%, 21% respectively and were significantly different (all pairwise comparisons p<0.0001). The within-subject variability (CV) for the cortisol response at 60 minutes in six participants who received 500 µg Nasacthin003 on three separate occasions (studies S3 and S4) ranged from 1·0% to 11·2% for plasma cortisol, 1·1% to 31·4% for salivary

318 cortisol and 3·1% to 23·4% for salivary cortisone (figure 2). Additionally we assessed
319 the within-participant variability of plasma cortisol by calculation of a repeatability
320 coefficient, which was 70·6 nmol/L at 30 minutes, such that two observations on the
321 same individual would be expected to fall within -70·6 and +70·6 nmol/L plasma
322 cortisol for 95% of the time. At 60 minutes the repeatability coefficient was 104·6
323 nmol/L. The repeatability coefficients calculated for the 60-minute concentration of
324 salivary cortisol and cortisone were 10·3 and 21·1 nmol/L respectively.

325

326 **Nasacthin003 in children (S5) (figure 3):** All 24 participating children received 500
327 µg Nasacthin003. Half received 1 µg (low-dose) intravenous Synacthen as their
328 comparator and the other half 145 µg/m^2 (high-dose) intravenous Synacthen and the
329 results were compared to those in adults from studies S2 and S3 (table 3). We found no
330 significant difference in the peak, 30- and 60-minute plasma cortisol between adults
331 and children for 500 µg Nasacthin003 or either of the two intravenous Synacthen doses.
332 There was no significant difference in the 60-minute plasma cortisol between the 500
333 µg Nasacthin003 formulation compared with 250 µg intravenous Synacthen with mean
334 difference (95% confidence interval): -15·9 nmol/L (-46·1, 14·4) p=0·26. Similarly we
335 found no difference at 60 minutes when measuring the glucocorticoid response in
336 saliva: mean difference (95% confidence interval): -3·8 nmol/L (-7·7, 0·2) p=0·06 for
337 salivary cortisol and -5.8 nmol/L (-14·7, 3·1) p = 0·17 for salivary cortisone.

338

339 **Absolute bioavailability of Nasacthin003 (S5) (table 4, figure 4)**: A summary of the
340 pharmacokinetic results from the paediatric cohort are shown in table 4. Our calculated
341 absolute bioavailability of 500 µg Nascthin003 against intravenous 145 µg/m^2

Synacthen was 0·143 (14·3%). The mean $AUC_{0\text{-}inf}$ for Nasacthin003 was over three-fold higher than the 1 µg intravenous Synacthen test (figure 4) but approximately a third that of the 145 µg/m^2 intravenous dose. Similarly, the mean Cmax for Nasacthin003 was higher than for 1 µg intravenous Synacthen (433 versus 245 pg/mL) but lower than the 145 µg/m^2 intravenous Synacthen test (433 versus 6702 pg/mL).

Safety: We found nasal tetracosactide formulations to be well tolerated by adults and children. We administered a total of 70 doses of Nasacthin003 to 46 subjects (22 adults, 24 children) with no serious adverse events (SAEs). Whilst 32% of participants did not report adverse events, the 68% who did experienced events that were anticipated following nasal drug administration; specifically watery eyes, coughing, sneezing, and a vinegary taste (acetic acid is a constituent of the drug product). These were all mild, with full and rapid resolution, and considered to be treatment-related. In addition, 12 doses of Nasacthin001, 12 doses of Nasacthin002, and 11 doses of 25 µg and 100 µg Synacthen were all administered intranasally with no SAEs reported. On post-study questionnaires the majority of participants (77%) reported nasal administration to be "easy" or "very easy", receiving the nasal drug as "no problem" or "slightly unpleasant" (86%) and "much the same", "better", or "much better" compared to the intravenous administration (71%).

Discussion

Over our four open-label multi-arm, sequence-randomised, crossover, pharmacodynamic studies we found 500 µg Nasacthin003 to demonstrate an equivalent plasma cortisol response to the intravenous SST at 60 minutes. Our repeatability study

demonstrated between and within-individual reproducibility of the test and the glucocorticoid response in children was indistinguishable from that of adults.

Our early studies tested six different doses from four formulations of tetracosactide. The response threshold required for maximal adrenal stimulation was not reached with intranasal doses derived from the commercially available intravenous formulation at 25 and 100 µg. The current 250 µg SST is widely recognised to deliver a supraphysiological adrenal stimulus. Studies attempting to quantify the ACTH level required to maximally stimulate the adrenal gland are decades old and hampered by assay issues but quote between 60-80 pg/ml, approximately half the endogenous peak values seen in healthy subjects in the early morning.[9,22] This is considerably lower than the levels generated following intravenous administration with 250 µg Synacthen (1580-66,000 pg/ml) but closer to those measured following administration of 1 µg (3·5-1920 pg/ml), leading some to advocate the 1 µg test as more physiological.[9,22] Groups comparing the low-dose and high-dose SST have demonstrated an equivalent cortisol response at 30 minutes, indicating that 1 µg is adequate to achieve maximal adrenal stimulation, but responses diverge thereafter, thought to be due to continued adrenocortical stimulation with higher doses.[8-10,14] In our studies, the addition of the nasal drug enhancer, chitosan, and dose escalation both significantly improved absorption of tetracosactide and the resultant glucocorticoid response, yielding plasma tetracosactide levels sufficient to maximally stimulate the adrenal cortex. Additionally our PK analysis demonstrated tetracosactide levels in excess of those seen after stimulation with 1 µg of intravenous Synacthen. The 500 µg Nasacthin003 dose was chosen for further study as it demonstrated that it exceeded the threshold for maximal adrenal stimulation, with no difference in plasma cortisol levels at 60 minutes when compared to the 250 µg intravenous test.

The SDs and CVs for the cortisol response at 60 minutes for 500 µg Nasacthin003 were higher than for 250 µg intravenous Synacthen. To our knowledge between-participant repeatability has not previously been reported for the 250 µg intravenous SST. We demonstrated CVs at 60 minutes post-stimulation, both within and between-participants, for the intranasal preparation which were better than that previously published for low-dose intravenous Synacthen. Within-participant CVs ranged from 3·0-16·4% in one study and 46·7-57·2% in another, with between-participant 28·0-48·6%.[23,24]

Analogues of ACTH, including ACTH(1-24) (Synacthen/Cosyntropin) have been administered nasally in historical studies for treatment, but the investigation for diagnostic purposes is novel.[18,19] Synacthen is inactivated in the gastrointestinal tract by proteolytic enzymes and therefore needs parenteral administration. It is a large polar molecule with a molecular mass of 2932 g/mol. These are not ideal properties for a nasal drug, which tend to be small and lipophilic. Drugs with a molecular weight below 1000 g/mol generally do not require adjuvants for effective absorption but larger peptide molecules benefit from the increased drug residence times, by slowing mucociliary clearance, and modifying transmembrane transportation.[17] The nasal route for proteins of a similar size has been previously investigated. For example, desmopressin (1069 g/mol) has a bioavailability of approximately 10% of the intravenous route and salmon calcitonin (3455 g/mol) has a relative bioavailability of between 3·9% and 7·9% when delivered with the enhancer sodium tauro-24,25-dihydrofusidate.[25] Improved Synacthen absorption with the addition of nasal drug enhancers (sodium glycocholate and bacitracin) has been demonstrated in a murine study but the combination of tetracosactide and chitosan to promote nasal absorption is novel.[20]

The method of diagnosis of AI by the SST has been much debated. Points of discussion include: what dose of Synacthen to administer, which cortisol assay platform to analyse on and what sampling times and diagnostic criteria to employ. Our recently published international survey on SSTs reported widespread variation including cortisol sampling timings and interpretative thresholds.[3] Our non-invasive test affords the opportunity for research in large cohorts where controversies exist over diagnosis and management, in particular monitoring and testing of patients on glucocorticoids at risk of AI. Termed tertiary AI this is now thought to be the commonest cause of AI in the Western world.[2] Nearly 1% of the UK adult population are on oral glucocorticoids at any one time and 4·5% of UK children are prescribed inhaled glucocorticoids, 10% above the recommended dose.[26,27] Biochemical evidence of AI has been reported in up to 40% of children taking inhaled glucocorticoids.[28-30] The potential global clinical utility of a test that can be administered in the outpatient setting or the community offers the potential for cost savings by negating the need for day case admission to hospital.

The relationship between plasma cortisol and salivary cortisol and cortisone has been widely published.[13-15] Salivary glucocorticoid estimation is growing in popularity, as salivary glucocorticoids reflect plasma cortisol and sampling is more pleasant for patients. Late night salivary cortisol testing is a first line investigation in Cushing Syndrome.[11] Cortisone is the more abundant glucocorticoid in saliva as plasma free cortisol and salivary cortisol are rapidly converted to inactive cortisone by 11ß-hydroxysteroid dehydrogenase type 2, making it better suited for the diagnosis of AI.[13,15,16] Sixty minutes is the optimal timing of salivary glucocorticoid sampling following stimulation with intravenous Synacthen.[14] The salivary cortisol and cortisone responses in children were similar between Nasacthin003 and intravenous Synacthen but slightly lower in adults. This may represent a dose effect in adults although, as

discussed above, the evidence suggests Nasacthin003 exceeds the threshold for maximal stimulation. It will be important, when introducing the non-invasive intranasal test into clinical practice, to perform clinical trials to define normal ranges for salivary cortisol and cortisone and document the sensitivity and specificity to diagnose adrenal insufficiency.

There are a number of limitations of our studies. All our studies were conducted in healthy adult and child volunteers who were dexamethasone suppressed. However, this conferred considerable advantage; uniformity between subjects and a clean baseline from which to assess response to tetracosactide formulations, and perform PK modelling. We acknowledge the lack of a patient cohort, although our intention was to demonstrate equivalence with a diagnostic agent that has been in widespread use for more than 30 years, not redefine the SST. We employed different assay platforms for plasma cortisol (immunoassay) and the salivary glucocorticoids (LCMS). This was pragmatic and reflected analytical techniques employed in clinical practice. We used an in-house assay for plasma cortisol analysis and sent salivary samples to a laboratory with significant expertise in salivary steroid analysis, as this was not available at the time in our institution. In order to be able to combine the results the same assays were used for all samples across the five studies.

In conclusion, we have developed a non-invasive alternative to the SST, with the administration of intranasal 500 µg Nasacthin003 and measurement of either salivary cortisone or cortisol at baseline and 60 minutes. Nasacthin003 was well tolerated and in 70 doses administered to 46 participants only minor, short-lived, and anticipated adverse events were experienced. The new test could positively impact on the growing demand for adrenal function testing. It could be conducted globally in community and

465 outpatient settings, with potential cost savings, and reduced healthcare burden to
466 patients.
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Author contributions:

CJE, NPW, TNJ and RJR designed the studies. CJE, RV and ASC enrolled participants and conducted the studies. EHK validated and performed the tetracosactide assay. BK performed the salivary glucocorticoid analysis. TNJ, RNT, CJE, RV and ASC analysed the data. CJE wrote the paper and all authors contributed to reviewing and editing the manuscript.

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Figure legends:

Table 1. CONSORT TABLE: Overview of the five studies.

Table 2: Maximum glucocorticoid responses to different doses and formulations of tetracosactide administered in studies 1 (S1) and 2 (S2). Data displayed as mean and standard deviation (SD).

Table 3: Summary of adult and paediatric glucocorticoid response following tetracosactide challenge by single dose administration of 250 µg or 1 µg intravenous Synacthen or 500 µg Nasacthin003.

Table 4: Summary of paediatric pharmacokinetic parameters for plasma tetracosactide following administration of intravenous Synacthen and Nasacthin003 in Study S5. $AUC_{0\text{-}inf}$, area under the concentration-time curve extrapolated to infinity; $AUC_{0\text{-}Last}$, area under the concentration-time curve to last measurable concentration; C_{max} maximum plasma concentration; CI, confidence interval; F, bioavailability; GM, geometric mean; T_{max}, time of maximum concentration.

Figure 1. Mean plasma cortisol (1a), salivary cortisol (1b) and salivary cortisone (1c) concentration over time following single dose administration of 250 µg intravenous Synacthen (red line), 500 µg Nasacthin003 (blue line) and 1000 µg Nasacthin003 (green line) in dexamethasone-suppressed healthy adult male participants. SD shown as error bars.

Figure 2. Individual plasma cortisol concentration over time graphs following three separate single dose administrations of 500 µg Nasacthin003 in dexamethasone-suppressed healthy adult male participants.

Figure 3. Mean plasma cortisol (3a), salivary cortisol (3b) and salivary cortisone (3c) concentration over time following single dose administration of 250 µg intravenous Synacthen (red line) and 500 µg Nasacthin003 (blue line) in dexamethasone-suppressed healthy children. SD shown as error bars.

Figure 4: Mean plasma tetracosactide concentration over time following single dose administration of 1 µg intravenous Synacthen (purple line) and 500 µg Nasacthin003 (blue line) in dexamethasone-suppressed healthy paediatric subjects. SD shown as error bars.

Table 1. CONSORT TABLE: Overview of the five studies.

Study Identifier, Purpose	**Study Design[1] & population**	**Enrolment: Recruitment & eligibility screening**	**Test Product[3]; Dosage; Route of Administration**	**Number (N) completing study**	**Number (N) included in final analysis[6]**
Study 1 (S1) Formulation development	Open-label, multi-arm, crossover, PK Healthy adult males	Recruitment target = 12 Screened for eligibility = 13[2]	Synacthen: single nasal dose, 25 µg and 100 µg single iv dose, 1 µg	 N = 11[4] N = 12	 N = 10 N = 12
Study 2 (S2) Formulation optimisation	Open-label, multi-arm, crossover, PK Healthy adult males:	Recruitment target = 12 Screened for eligibility = 12	Nasacthin001, single nasal dose, 100 µg Nasacthin002, single nasal dose, 500 µg Nasacthin003, single nasal dose, 500 µg Synacthen, single iv dose, 1 µg	N = 12 N = 12 N = 10[5] N = 11[5]	N = 10 N = 12 N = 8 N = 10
Study 3 (S3) Dose response	Open-label, multi-arm, crossover, PK Healthy adult males	Recruitment target = 12 Screened for eligibility = 12	Nasacthin003, single nasal dose, 500 µg Nasacthin003, single nasal dose, 1 mg Synacthen, single iv dose, 250 µg	N = 12 N = 12 N = 12	N = 11 N = 12 N = 12
Study 4 (S4) Repeatability	PK repeatability Healthy adult males from S3	Recruitment target = 6	Participants received two further doses of: Nasacthin003, single nasal dose, 500 µg	 N = 6	 N = 6
Study 5 (S5) Paediatric study	Open-label, multi-arm, crossover, PK Healthy children	Recruitment target = 24 (12F) Screened for eligibility = 36	Nasacthin003, single nasal dose, 500 µg Synacthen, single iv dose, 1 µg Synacthen, single iv dose, 250 µg	N = 24 N = 12 N = 12	N = 23 N = 9 N = 11

1. Order of visits was randomised for studies S2, S3 and S5; visits separated by a minimum of one week.
2. Participant screened but excluded due to mild asthma
3. Nasacthin001, 100 µg tetracosactide and chitosan; Nasacthin002, 500 µg tetracosactide; Nasacthin003, 500 µg tetracosactide and chitosan.
4. Participant failed to attend subsequent visits
5. Participants removed from study due to mild gastrointestinal side-effects to dexamethasone
6. Number of participants completing the study and included in final data analyses. Dexamethasone suppression confirmed by baseline undetectable/low cortisol (<50 nmol/L), and subjects excluded from analysis if not adequately suppressed (13 of the total 175 participant visits).

Table 2: Maximum glucocorticoid responses to different doses and formulations of tetracosactide administered in studies 1 (S1) and 2 (S2). Data displayed as mean and standard deviation (SD).

Maximum concentration nmol/L			Plasma cortisol	Salivary cortisol	Salivary cortisone
Study	**Dose**				
S1	**Intranasal Synacthen 100 µg**	**N**	9	10	10
		Mean	159.6	1.1	8.1
		SD	95.0	0.6	6.5
	Intranasal Synacthen 25 µg	**N**	10	10	10
		Mean	63.9	1.0	3.5
		SD	57.7	0.5	2.8
	Intravenous Synacthen 1 µg	**N**	11	11	11
		Mean	388.2	7.2	29.1
		SD	48.6	4.0	7.7
S2	**Nasacthin001 100 µg**	**N**	11	11	11
		Mean	336.1	8.6	34.7
		SD	160.8	7.2	22.8
	Nasacthin002 500 µg	**N**	12	12	12
		Mean	401.6	16.2	55.2
		SD	183.7	14.3	38.0
	Nasacthin003 500 µg	**N**	8	8	8
		Mean	648.7	28.7	82.1
		SD	141.0	12.9	23.9
	Intravenous Synacthen 1 µg	**N**	10	10	10
		Mean	391.4	10.2	39.5
		SD	90.5	4.6	13.9

Table 3: Summary of adult and paediatric glucocorticoid response following tetracosactide challenge by single dose administration of 250 µg or 1 µg intravenous Synacthen or 500 µg Nasacthin003.

Parameter (all nmol/L)		Nasacthin003, 500 µg intranasal			Synacthen, 250 µg intravenous			Synacthen, 1 µg intravenous		
		Plasma Cortisol	Salivary Cortisol	Salivary Cortisone	Plasma Cortisol	Salivary Cortisol	Salivary Cortisone	Plasma Cortisol	Salivary Cortisol	Salivary Cortisone
Peak glucocorticoid concentration (nmol/L)	**Adult** Mean (±SD)	547 (±106)	25·4 (±9·2)	64·7 (±19·4)	615 (±51)	35·8 (±10·7)	78·1 (±11·5)	388 (±67)	8·5 (±4·5)	33·7 (±11·6)
	Children Mean (±SD)	568 (±81)	30·7 (±9·0)	65·9 (±14·9)	626 (±54)	37·2 (±9·0)	80·0 (±15·9)	401 (±80)	12·8 (±4·9)	40·3 (±10·9)
	All Mean (±SD)	556 (±97)	27·7 (±9·5)	65·2 (±17·7)	620 (±53)	36·5 (±9·9)	79·0 (±13·8)	392 (±72)	9·8 (±5·0)	35·6 (±11·8)
Time at peak glucocorticoid concentration (mins)	**Adult** Median Range	75 40–120	90 40–120	90 40–180	120 120–120	120 120–120	120 120–120	30 15–40	40 20–150	40 30–60
	Children Median Range	75 40–120	75 50–120	90 50–120	105 90–120	120 90–120	120 90–120	40 30–50	40 30–50	40 30–50
	All Median Range	75 40–120	82.5 40–120	90 40–180	120 90–120	120 90–120	120 90–120	30 15–50	40 20-150	40 30–60
Mean concentration at 30 minutes Mean (±SD)		398 (±61)	10·6 (±4·2)	27·0 (±10·1)	428 (±53)	12·1 (±4·4)	31·2 (±9·4)	379 (±61)	8·2 (±4·0)	27·6 (±10·8)
Mean concentration at 60 minutes Mean (±SD)		511 (±88)	21·6 (±7·1)	54·4 (±13·4)	531 (±59)	24·9 (±7·5)	59·0 (±12·1)	270 (±75)	5·5 (±4·1)	28·5 (±10·4)
Number of subjects		Adult: N=19 (31 doses) Children: N=23			Adult: N=12 Children: N=11			Adult: N=21 Children: N=9		

Table 4: Summary of paediatric pharmacokinetic parameters for plasma tetracosactide following administration of intravenous Synacthen and Nasacthin003 in Study S5.

$AUC_{0\text{-}inf}$, area under the concentration-time curve extrapolated to infinity; $AUC_{0\text{-}Last}$, area under the concentration-time curve to last measurable concentration; C_{max} maximum plasma concentration; CI, confidence interval; F, bioavailability; GM, geometric mean; T_{max}, time of maximum concentration.

PK parameter GM (95% CI); Median (range) for T_{max}	**Nasacthin003**	**Synacthen**	
	500 µg, intranasal	250 µg, intravenous	1 µg, intravenous
Tetracosactide C_{max} (pg/mL)	433 (324–602)	6702 (4346–10335)	245 (199–302)
$AUC_{0\text{-}Last}$ (pg/ml*min)	16672 (12845–20192)	75329 (45537–124611)	3409 (2691–3904)
$AUC_{0\text{-}inf}$ (pg/ml*min)	20934 (16274–24156)	77058 (46899–126611)	6212 (4789–8058)
$F_{0\text{-}inf}$	0·143 (0·082–0·249)	-	-
T_{max} (mins) Median (range)	10 (5–20)	-	-

Figure 1. Mean plasma cortisol (1a), salivary cortisol (1b) and salivary cortisone (1c) concentration over time following single dose administration of 250 µg intravenous Synacthen (red line), 500 µg Nasacthin003 (blue line) and 1000 µg Nasacthin003 (green line) in dexamethasone-suppressed healthy adult male participants. SD shown as error bars.

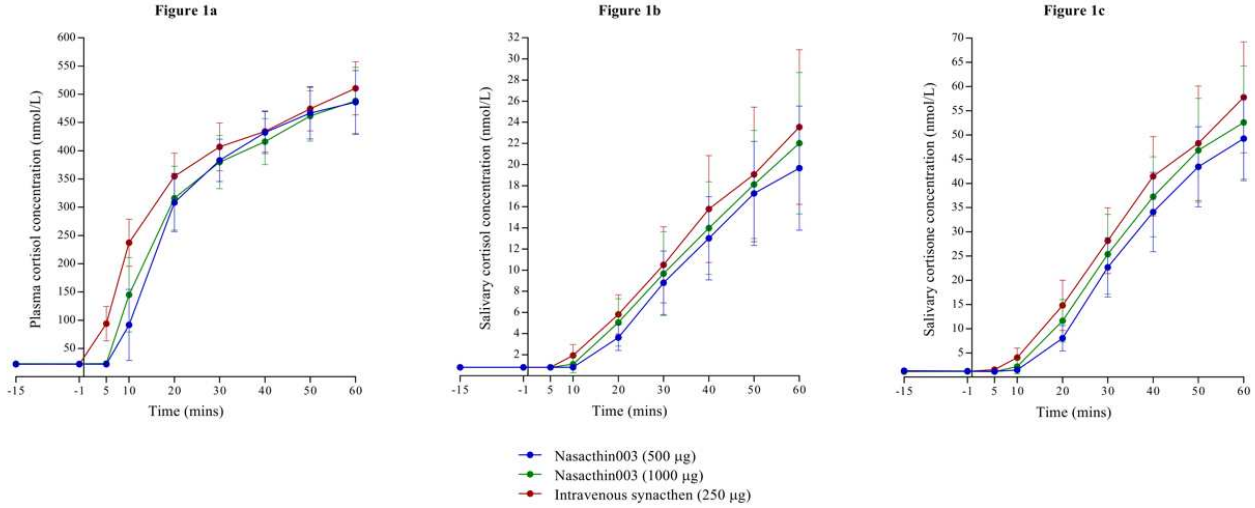


Figure 2. Individual plasma cortisol concentration over time graphs following three separate single dose administrations of 500 µg Nasacthin003 in dexamethasone-suppressed healthy adult male participants.

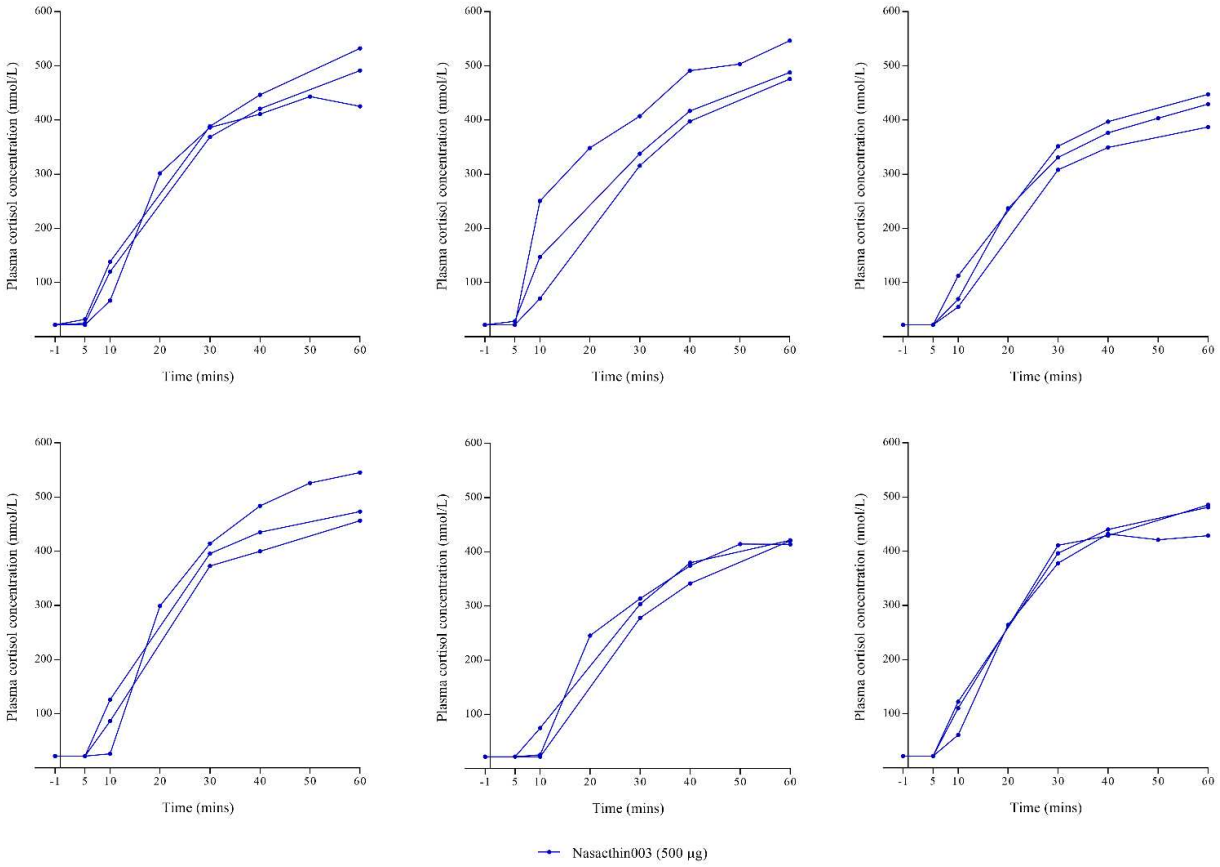


Figure 3. Mean plasma cortisol (3a), salivary cortisol (3b) and salivary cortisone (3c) concentration over time following single dose administration of 250 µg intravenous Synacthen (red line) and 500 µg Nasacthin003 (blue line) in dexamethasone-suppressed healthy children. SD shown as error bars.

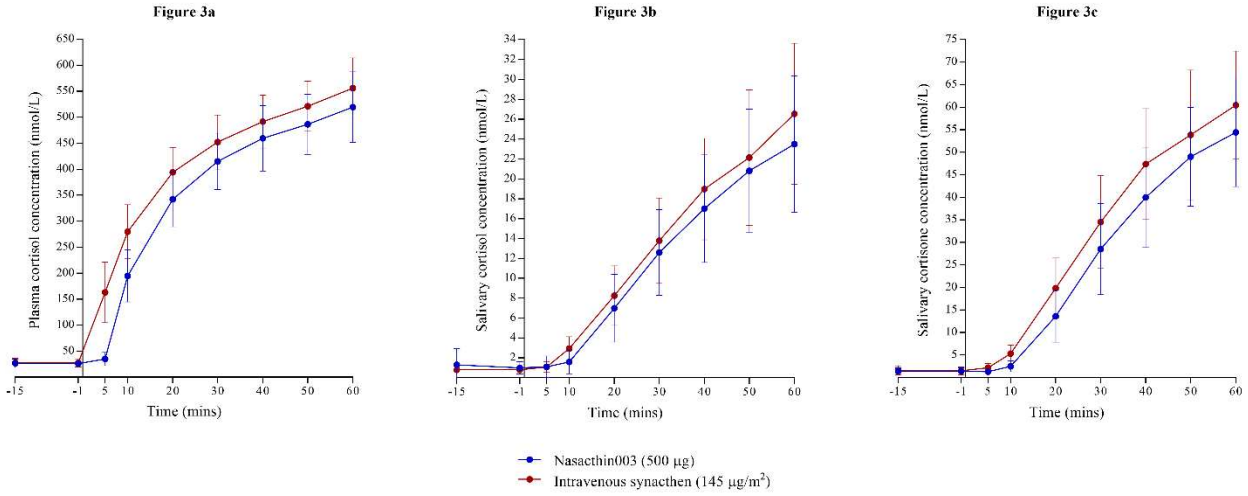
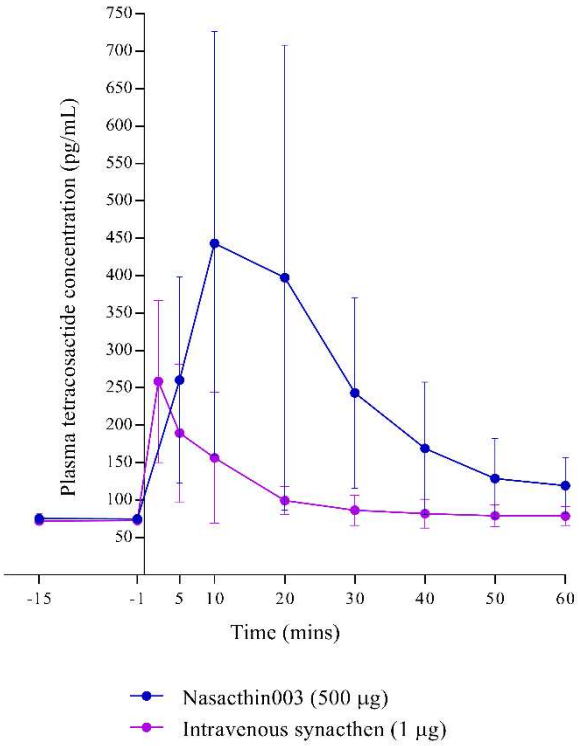


Figure 4: Mean plasma tetracosactide concentration over time following single dose administration of 1 µg intravenous Synacthen (purple line) and 500 µg Nasacthin003 (blue line) in dexamethasone-suppressed healthy paediatric subjects. SD shown as error bars.



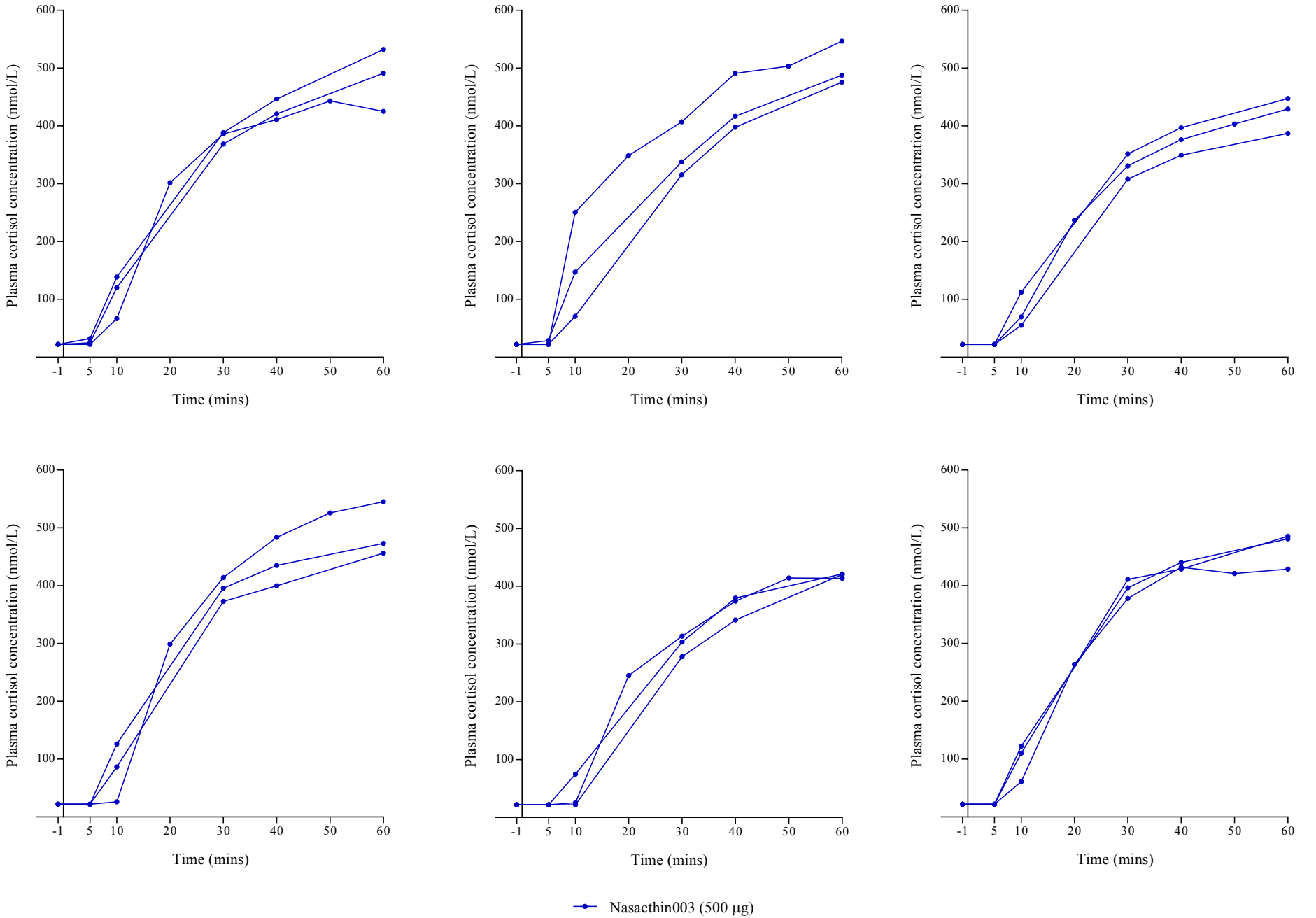


Figure 1

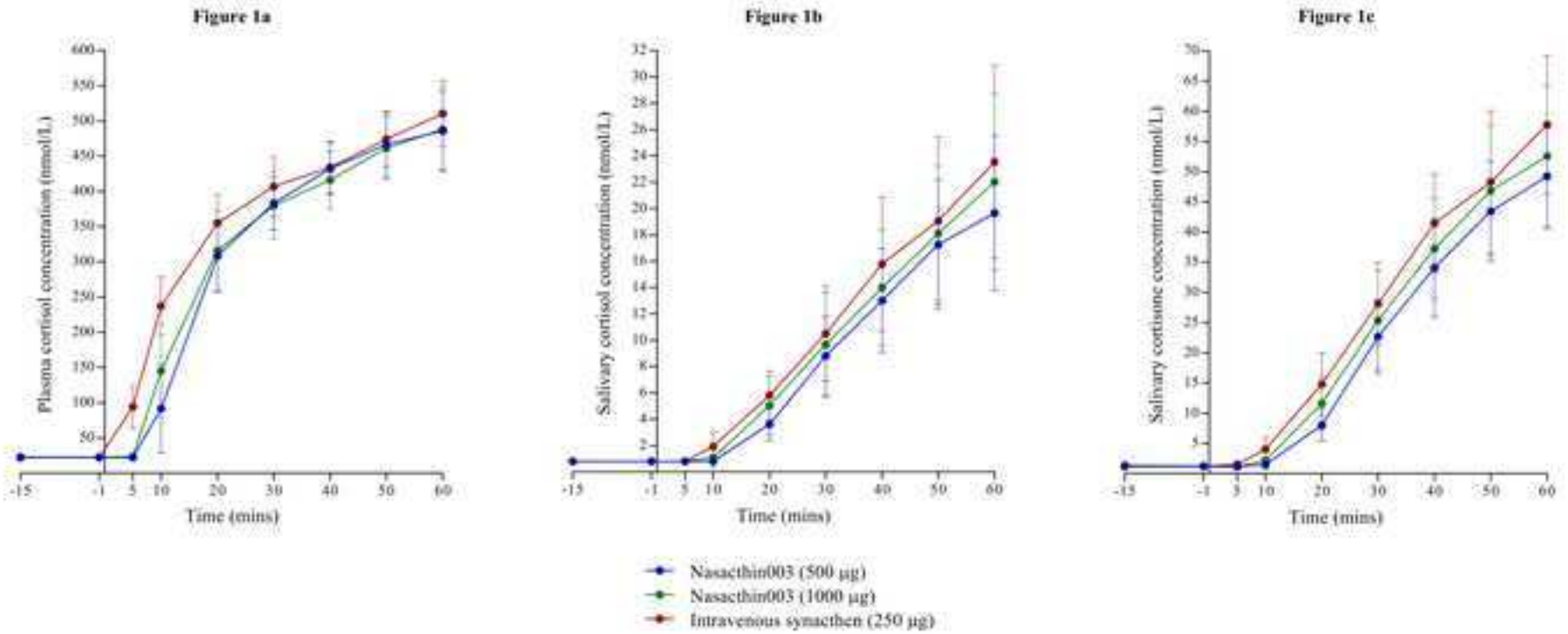


Figure 3

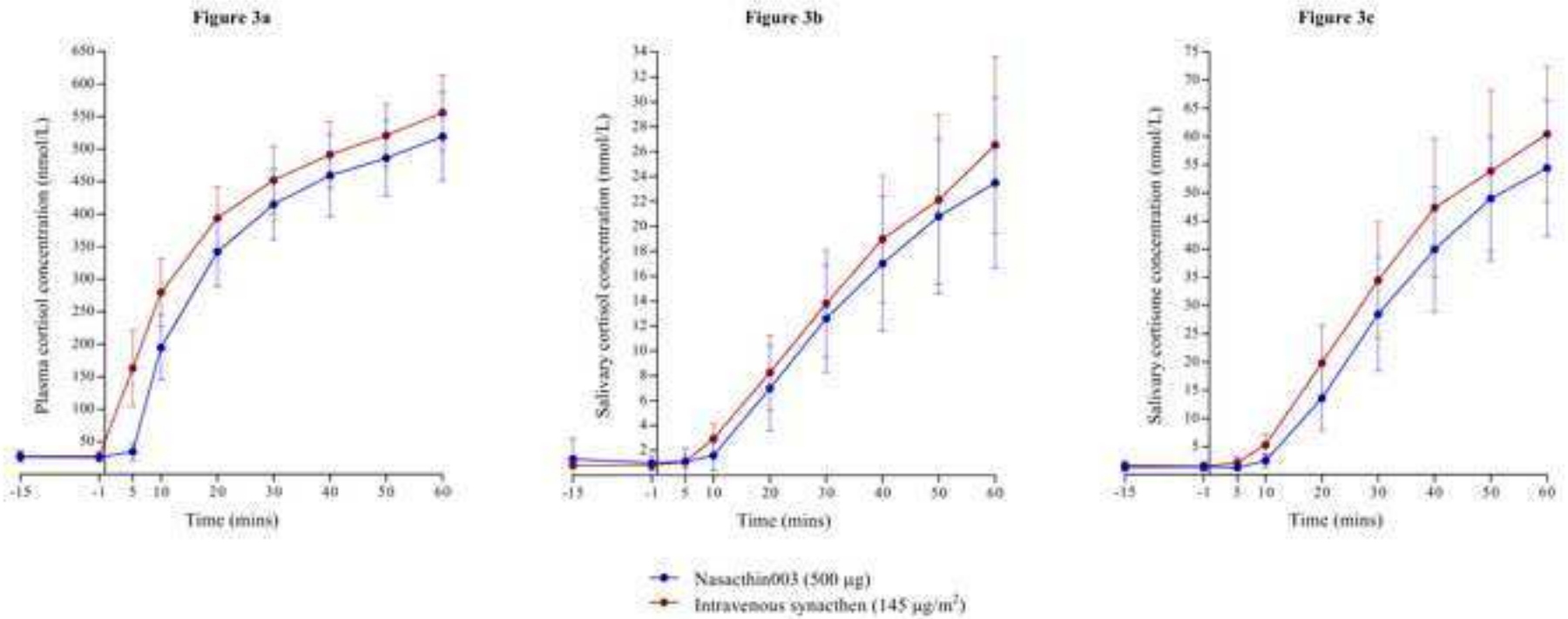


Figure 4

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